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Maple Sirup XII

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XII. Effect of Zinc on the Growth of Microorganisms in Maple Sap

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A recent study in this laboratory demonstrated the effect of controlled fermentation of maple sap on the quality of the sirup obtained (Naghski *et al.*, 1957). These fermentations were carried out in 50-L glass carboys. When the glass containers were replaced by galvanized, 10-gal cans, variable results were observed. It was noted that, in new cans, several of the inocula diminished almost to sterility within a few days, whereas in older cans, growth progressed at about the usual rate. This germicidal effect of the cans was tentatively attributed to the zinc coatings.

The oligodynamic effect of metals, including zinc, on microorganisms received considerable attention several decades ago. Inhibition by zinc has been reported for many types of bacteria (Hotchkiss, 1923; Colley, 1931; Winslow and Haywood, 1931; Perlman, 1945; Hughes, 1948; Lees, 1948; Svec, 1948; Abelson and Aldous, 1950; MacLeod and Snell, 1950; Feeney, 1952; MacLeod, 1954) and fungi (Seifriz and Uraguchi, 1941; Tomlinson *et al.*, 1950; Miller and McCallan, 1957). As with many inorganic inhibitors, zinc in dilute concentration can be stimulatory to growth (Hotchkiss, 1923; Colley, 1931; Winslow and Haywood, 1931).

This study was initiated to determine whether or not zinc ions from the surfaces of galvanized containers could dissolve in sufficient concentration to inhibit the organisms inoculated into maple sap.

Experimental Methods

Cultures. Five bacterial and two yeast strains, isolated previously from naturally fermenting sap, were employed in this study. Preliminary taxonomic studies have been described elsewhere and temporary generic designations assigned to some of these strains (Naghski *et al.*, 1957). Identification of one yeast strain as *Cryptococcus albidus* was made by L. J. Wickerham of the Northern Utilization Research and Development Division at Peoria, Illinois. The following designations were used for the strains included in this study: *Bacterium*-587, *Pseudomonas*-11, *Pseudomonas*-25, *Flavobacterium*-583, *Achromobacter*-385, yeast-M6, *Cryptococcus albidus*-496. Stock cultures of all organisms were maintained on nutrient agar slants.

Preparation of inocula. Slants of tryptone glucose yeast extract (TGY) agar were inoculated from stock cultures. After incubation for 24 hr at 27 C, growth was washed from the surface with sterile distilled water and diluted to the desired concentration. Mixed inocula were made by combination of suspensions of the desired strains. Initial concentrations were estimated immediately following inoculation of the suspending medium by appropriate dilution, plating with TGY agar, and incubation for 2 days at 27 C.

Preparation of maple sap. Cans of sterile, frozen maple sap were thawed as described previously (Naghski *et al.*, 1957) and distributed into the several types of containers investigated. Maple sap with specific concentrations of several zinc salts was prepared in Erlenmeyer flasks and sterilized by autoclaving before inoculation.

Incubation. Inoculated media were incubated in the cold room at 0.5 to 2.5 C unless specified otherwise. Length of incubation for each experiment varied and is given in the section dealing with results. Concentration of organisms after various incubation times was estimated by plating with TGY agar.

Containers. Five-gal Pyrex carboys were sterilized in the autoclave. Galvanized, 10-gal garbage cans were sanitized by washing for 2 hr with flowing steam. Cleaning of used galvanized containers (which had acquired a protective surface coating) prior to sanitizing will be described in the appropriate section of the results.

Chemicals. All compounds employed were of reagent grade and were obtained from commercial sources.

Analytical methods. Zinc was determined polarographically.

Results

The effect of the type of container surface on the growth of a mixed population in maple sap held at 0.5 to 2.5 C was studied with an inoculum composed of *Pseudomonas*-25, *Flavobacterium*-583, *Bacterium*-587, *Achromobacter*-385, and *Cryptococcus albidus*-496 and is shown in figure 1. The progressive decline in viable count of microorganisms incubated in the new galvanized cans contrasts sharply with the normal growth curves observed for this population incubated in a glass container or in used galvanized cans. The alkali-

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washed, used container was cleaned with a 35 per cent solution of NaOH and rinsed thoroughly with distilled water before sanitizing. It is apparent that washing with strong alkali did not alter the protective surface film sufficiently to permit solution of the germicidal factor.

Figure 2 demonstrates that the character as well as the cleanliness of the metal surface determines the effect of the container on the inoculum. Sap was inoculated with a mixture of *Pseudomonas*-11 and *Flavobacterium*-583; distributed in a glass carboy, a used metal container, a new galvanized container, and an acid-washed, used galvanized container; and incubated at 0.5 to 2.5 C for 8 days. Periodically during incubation, counts were made and soluble zinc estimated in the saps. The acid-washed container was cleaned with a 6 per cent solution of sulfamic acid and rinsed thoroughly with distilled water before sanitizing with steam.

Typical growth curves were noted in the glass container and in the used, untreated galvanized can, whereas a gradual decrease in the initial bacterial count in the acid-treated can was observed. Effective removal of the protective film had restored the can surface to its original state as a supplier of the active substance. The bacterial count of the sap incubated in the new container revealed inhibition on the second day of incubation followed by a gradual decrease in number as incubation continued. The new can used in this experiment had a smooth surface and did not have the rough zinc crystal formation characteristic of the cans used in the first experiment (figure 1). Table 1 and figure 2 demonstrate that changes in bacterial counts can be linked to the presence of zinc ions dissolved from the container surfaces. It can be seen that inhibition of growth was exerted on the inoculum in the acid-washed can through the second day of inhibition when the concentration of dissolved zinc was about 11 ppm (table 1). As the dissolved zinc increased, the toxic effect of zinc became apparent from the decrease in bacterial count. In the new container, growth progressed normally for 2 to 3 days in the presence of about 6 ppm dissolved zinc. As the zinc concentration increased to a

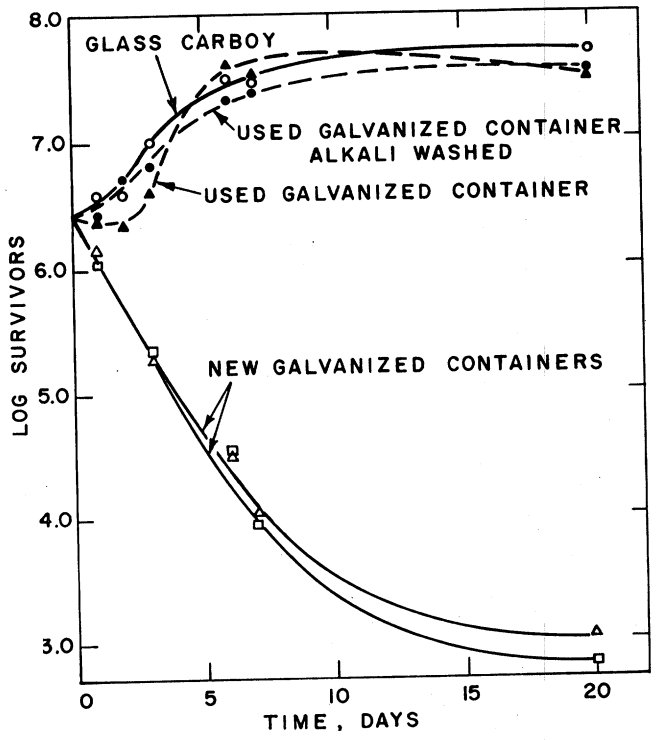

Figure 1. The growth of a mixed inoculum in maple sap held at 0.5 to 2.5 C in different containers.

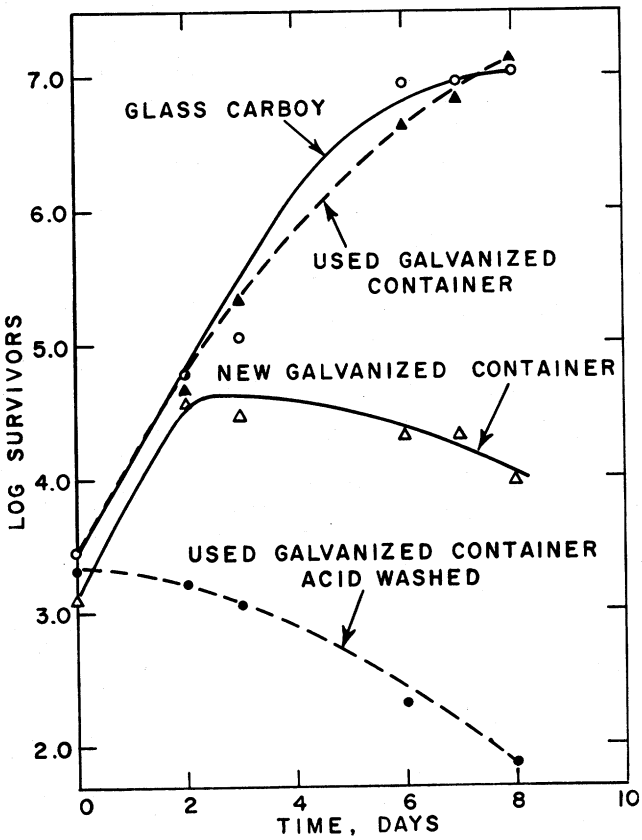

Figure 2. The growth of a mixed inoculum in maple sap held at 0.5 to 2.5 C in different containers.

TABLE 1

Zinc dissolved from various containers during fermentation of inoculated maple sap held at 0.5 to 2.5 C

Type of Container	Days of Incubation				
	0	2	3	6	8
	Zinc, ppm				
Used galvanized can (untreated)	0	2.7	2.7	3.1	1.8
Used galvanized can (acid-washed)	0	11.3	11.3	16.2	20.9
New galvanized can	0	5.4	6.7	11.1	9.7
Glass carboy	0	0	0	0	0

maximum of 10 to 11 ppm, the slight bactericidal effect was observed.

The toxic effect of several zinc salts (acetate, chloride, nitrate, and sulfate) on *Pseudomonas*-25 in maple sap incubated at room temperature was investigated. The pH of the sap after supplementation was 6.50 ± 0.20 and did not affect growth of this organism. The numbers of survivors in the presence of various concentrations of zinc salts were estimated after incubation for 24, 48, and 72 hr. The results after incubation for 24 hr are shown in figure 3. Counts made after 48 and 72 hr of incubation gave survival pictures similar to those at 24 hr, but with fewer survivors. In general, it can be said that all zinc salts tested exerted similar toxic effects.

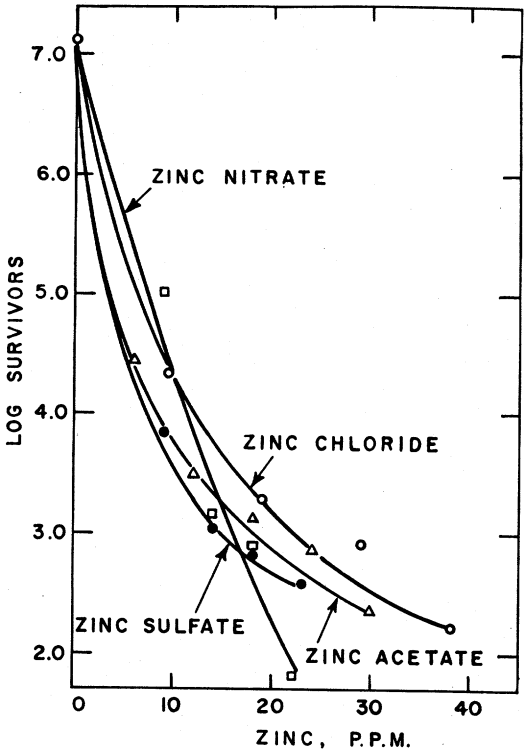

Figure 3. The effect of various zinc salts on the growth of *Pseudomonas*-25 in maple sap held for 24 hr at room temperature.

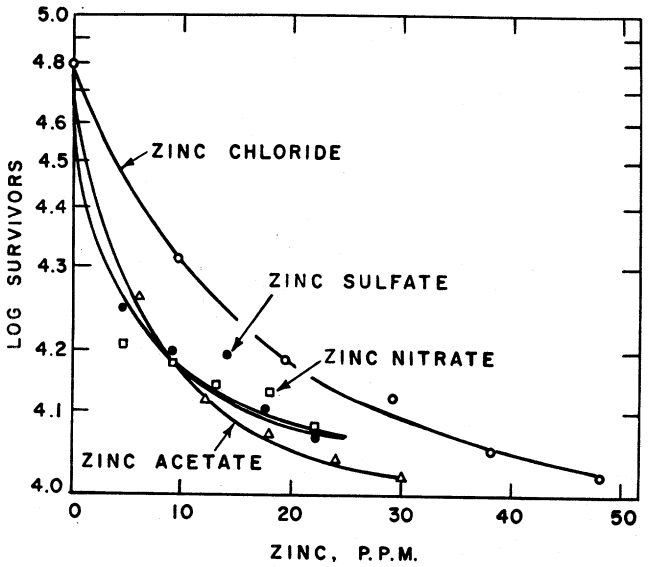

Figure 4. The effect of various zinc salts on the growth of *Pseudomonas*-25 in maple sap held for 24 hr at 0.5 to 2.5 C.

Since *Pseudomonas*-25 is capable of growth under psychrophilic conditions, and since maple sap flows and is held at near-freezing temperatures, the effect of zinc salts was studied after 1 and 7 days of incubation at 0.5 to 2.5 C. The results of these experiments are shown in figures 4 and 5.

A gradual decrease in bacterial count was noted after 1 day of exposure to zinc salts in maple sap (figure 4). The extent of this decrease was more pronounced after 7 days. The spread of the curves seen in figure 5 suggests that the anions may also influence the germicidal effect of zinc against *Pseudomonas*-25.

Discussion

The collection of maple sap by the conventional method of a spile and bucket provides suitable conditions for the introduction of spoilage organisms. Since a high level of microbial activity results in production of sirup of lower quality, it is desirable either to prevent the entrance of microorganisms into maple sap or to control their activity.

Several years ago one maple processor employed new, large, galvanized cans to avoid the necessity of frequently emptying the smaller collection buckets. The sap, therefore, was held unprocessed for a longer period of

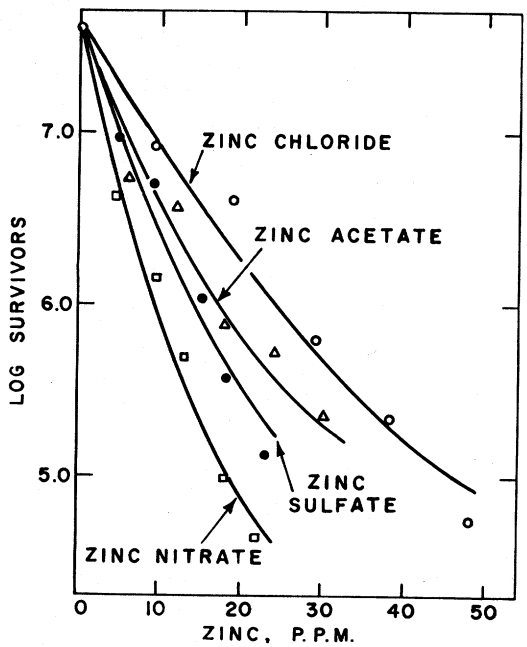

Figure 5. The effect of various zinc salts on the growth of *Pseudomonas*-25 in maple sap held for 7 days at 0.5 to 2.5 C.

time (1 to 2 weeks), contrary to good maple sap handling practices requiring sap to be processed within 1 day after issuing from the tree tissue. It was expected that this sap would have a relatively high bacterial count and yield lower grade sirups. However, sap collected in this manner proved to be low in count and gave high quality sirup.

From the data presented in figure 2 and table 1, it is seen that a concentration of 5 ppm zinc will inhibit multiplication of microorganisms inoculated into sap. With longer periods of incubation, this concentration can exert a bactericidal effect (figure 5). For shorter exposures, a concentration of 10 to 15 ppm zinc is toxic to the microorganisms (figures 3 and 4).

The rate of destruction by zinc is slower at low temperatures (figure 4) than at warm temperatures (figure 3). Since maple sap collection is carried out at near-freezing temperatures it might be surmised that a high concentration of zinc would be desirable to overcome the slow death rate. This is unnecessary, however, since inhibition of the population to a low level is sufficient to insure that spoilage of the sap is kept to a minimum.

When galvanized cans are used for the collection of maple sap, these containers must be carefully cleaned in order to remove the accumulated coating from the zinc surface.

Acknowledgments

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Summary

This study demonstrated that growth of microorganisms in maple sap can be inhibited by new or acid-cleaned, used galvanized containers. The bacteriostatic and bactericidal effects were correlated with the concentration of zinc ion dissolving from the container surface. Several zinc salts (acetate, chloride, nitrate, and sulfate) were toxic to the growth of a strain of *Pseudomonas* in maple sap stored under psychrophilic as well as mesophilic conditions.

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